Presence of AmpC beta-lactamases, CSA-1, CSA-2, CMA-1, and CMA-2 conferring an unusual resistance phenotype in Cronobacter sakazakii and Cronobacter malonaticus

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Abstract: Here we describe the presence of two very similar but unusual variants of AmpC cephalosporinase in each Cronobacter sakazakii and C. malonaticus isolates conferring resistance exclusively to first generation cephalosporins. During a survey on the antibiotic resistance patterns of C. sakazakii and C. malonaticus strains isolated from a milk powder production facility, originally two different phenotypes regarding the susceptibility/resistance for the two beta-lactam antibiotics ampicillin (amp) and cephalothin (ceph) were observed: (i) isolates being susceptible for both antibiotics (amp(S)/ceph(S)), and (ii) strains exhibiting susceptibility to ampicillin but resistance to cephalothin (amp(S)/ceph(R)). The latter phenotype (amp(S)/ceph(R)) was observed in the majority of the environmental strains from the facility. Analysis of whole genome sequences of C. sakazakii revealed a gene putatively coding for an AmpC beta-lactamase. Consequently, the ampC genes from both species and both phenotypes were subjected to a cloning approach. Surprisingly, when expressed in Escherichia coli, all transformants exhibited the amp(S)/ceph(R) phenotype regardless of (i) the phenotypic backgrounds or (ii) the AmpC amino acid sequences of the original strains from which the clones were derived. The novel AmpC beta-lactamases were designated CSA-1 and CSA-2 (from C. sakazakii) and CMA-1 and CMA-2 (from C. malonaticus). The observed variations in the minimum inhibitory concentration (MIC) levels for cephalothin (wt compared to transformants) suggest that this feature is a target of a yet unknown regulatory mechanism present in the natural Cronobacter background but absent in the neutral E. coli host.

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Here we describe the presence of two very similar but unusual variants of AmpC cephalosporinase in each *Cronobacter sakazakii* and *C. malonaticus* isolates conferring resistance exclusively to first generation cephalosporins. During a survey on the antibiotic resistance patterns of *C. sakazakii* and *C. malonaticus* strains isolated from a milk powder production facility, originally two different phenotypes regarding the susceptibility/resistance for the two beta-lactam antibiotics ampicillin (amp) and cephalothin (ceph) were observed: (i) isolates being susceptible for both antibiotics ($amp^S/ceph^S$), and (ii) strains exhibiting susceptibility to ampicillin but resistance to cephalothin ($amp^S/ceph^R$). The latter phenotype ($amp^S/ceph^R$) was observed in the majority of the environmental strains from the facility. Analysis of whole genome sequences of *C. sakazakii* revealed a gene putatively coding for an AmpC beta-lactamase. Consequently, the *ampC* genes from both species and both phenotypes were subjected to a cloning approach. Surprisingly, when expressed in *Escherichia coli*, all transformants exhibited the $amp^S/ceph^R$ phenotype regardless of (i) the phenotypic backgrounds or (ii) the AmpC amino acid sequences of the original strains from which the clones were derived. The novel AmpC beta-lactamases were designated CSA-1 and CSA-2 (from *C. sakazakii*) and CMA-1 and CMA-2 (from *C. malonaticus*). The observed variations in the minimum inhibitory concentration (MIC) levels for cephalothin (wt compared to transformants) suggest that this feature is a target of a yet unknown regulatory mechanism present in the natural *Cronobacter* background but absent in the neutral *E. coli* host.

Introduction

According to the List of Prokaryotic Names with Standing in Nomenclature (www.bacterio.net/allnamesac.html), the genus *Cronobacter* was proposed in 2008 and currently consists of seven species, which encompass organisms previously identified as *Enterobacter sakazakii*.[8,9] Members of this genus are considered opportunistic pathogens, and are associated with outbreaks and sporadic infections, particularly in low birth weight and premature infants.[1,12,13] Symptoms include bacteremia, necrotizing enterocoliti,s and meningitis, with fatality rates between 10% and 42%.[6] The prognosis for survivors is generally poor, and neurological delay and poor lifelong outcomes may occur in more than 74% of cases.[19] Antibiotic susceptibility testing, when available, showed that *Cronobacter* isolates were usually sensitive to ampicillin, the aminoglycosides, chloramphenicol, and third generation cephalosporins.[13] However, in a study by Lai,[10] cases of nosocomial *Cronobacter* infections were examined, thereby revealing that all of the isolates were uniformly resistant to ampicillin, cephazolin, and extended spectrum penicillins, and were not uniformly susceptible to the third generation cephalosporins.

Antimicrobial susceptibility testing on environmental *Cronobacter* spp. isolates, as performed in more recent studies, indicated susceptibility to ampicillin (and all cephalosporins), thus leading to speculations that *Cronobacter* wild type strains may lack a beta-lactamase.[4,7] Genera such as *Enterobacter*, *Citrobacter*, *Serratia*, *Proteus*, *Providencia*, and *Morganella* usually express a chromosomal, inducible group I AmpC type beta-lactamase that confers strong resistance to penicillins (including ampicillin and inhibitor combinations) and first to third generation cephalosporins.[11]

In this study, we analyzed *C. sakazakii* and *C. malonaticus* strains originating from a milk powder production facility and respective species type strains for their antimicrobial resistance against the two major beta-lactam antibiotics—ampicillin and cephalothin—in order to answer the question of the presence/expression of a beta-lactamase in *Cronobacter*.

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Material and Methods

Strains and antibiotic susceptibility testing

In a study by Müller *et al*.,[14] 139 *C. sakazakii* and *C. malonaticus* isolates from a milk powder production facility were collected and characterized using several typing methods. These isolates were examined in this study for their resistance against major antibiotics using the disk diffusion assay,[3] as well as by determining the minimum inhibitory concentration (MIC) values by means of the E-Test (Biomerieux) for four selected strains, two from the above mentioned study—*C. sakazakii* Su 92 and *C. malonaticus* Su 126—as well as two type strains—*C. sakazakii* ATCC 29544^T and *C. malonaticus* type strain LMG 23826^T. *Escherichia coli* DH5 alpha as well as *E. coli* DH5 alpha harboring the pCCR9 cloning vector were included in the study. *Cronobacter* strains and *E. coli* DH5 alpha were cultivated in LB, transformants and *E. coli* DH5 alpha harboring pCCR9[18] vector were grown in LB supplemented with tetracycline (50 mg/L) at 37°C overnight. Strains used in the study and respective susceptibility data are given in Table 1.

Table 1. Minimal Inhibitory Concentrations of Two Critical Antibiotics Observed in the Four Selected *Cronobacter* Strains, Their Respective *Escherichia coli* DH5 Alpha Transformants, the Cloning Host *E. coli* DH5 Alpha, and the Cloning Host Containing the Empty Cloning Vector pCCR9

Strains	*Ampicillin*[a]	*Cephalothin*[a]
C. sakazakii ATCC 29544^T wt	1	32
E. coli DH5 alpha + pCCR9::*ampC*$_{ATCC\ 29544T}$	4	> 256
C. sakazakii Su 92 wt	1	8
E. coli DH5 alpha + pCCR9::*ampC*$_{Su92}$	8	> 256
C. malonaticus Su 126 wt	1.5	32
E. coli DH5 alpha + pCCR9::*ampC*$_{Su126}$	3	48
C. malonaticus LMG 23826^T wt	0.5	3
E. coli DH5 alpha + pCCR9::*ampC*$_{LMG\ 23826T}$	4	128
E. coli DH5alpha	0.75	1.5
E. coli DH5 alpha + pCCR9	1.5	1.5

[a]Given in mg/L, scores: ≤8: susceptible, ≥32 resistant (CLSI, 2013).

Identification of a putative beta-lactamase gene in C. sakazakii *ES15 and primer design*

A database search was performed on the whole genome sequence available for *C. sakazakii* ES15 *(*GenBank: CP003312.1) and a coding sequence (CDS) annotated as putative beta-lactamase (*ampC*, GenBank: AFJ99606.1, genome position: 2.159.483–2.160.610) was identified. Analysis of the regions up- and downstream of this CDS revealed a putative promoter element. Based on these data, PCR primers (CampCf: 5′-TGA ACC AGA ACG GAT TAG CCC-3′, genome position: 2.160.772–2.161.793; and CampCr: 5′-CAG CGA TGC CGA CTT CAA CGC-3′, genome position: 2.159.338–2.159.359) were designed and employed in order to amplify this CDS in the four selected *Cronobacter* strains using the following conditions: 20 ng template DNA, 0.4 μM primers, 1 × AccuPrime (Invitrogen) buffer 2 (60 mM Tris-SO_4 [pH 8.9], 18 mM $[NH_4]_2SO$), 2 mM $MgSO_4$, 2 mM dGTP, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, thermostable AccuPrime^{TM} protein, 1% glycerol, 2 U AccuPrime Taq DNA Polymerase High Fidelity (Invitrogen). The following PCR conditions were employed: 94°C for 30 s followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 68°C for 120 s. DNA was extracted from LB cultures grown overnight using the Qiagen Blood and Tissue Kit (Qiagen). The 1309 bp amplicons were successfully amplified and sequenced using the above mentioned primers.

Sequencing and sequence analysis

Sequencing of the *ampC* amplicons and the respective transformant inserts was outsourced (Microsynth). Sequence analysis was performed using the CLC Main Workbench 5 software.

Cloning of ampC *CDS (+ promoter element)*

For cloning purposes, the above mentioned primers were extended by a *HindIII* (forward primer) and a *BamHI* (reverse primer) restriction enzyme recognition sequence. The amplicons were double digested, purified using the Qiagen PCR purification Kit. Vector pCCR9 DNA was obtained using the Qiagen Plasmid Midi Kit double digested with the above mentioned enzymes, (0.7% agarose) gel purified, and DNA was released employing the Qiagen Gel purification Kit. Vector-insert ligation was performed using the T4 ligase (New England Biolabs). Constructs were transformed in *E. coli* DH5 alpha cells by electroporation.

Accession numbers

The *ampC* gene sequences of the isolates included in this study were deposited in Genbank under the following numbers: *C. sakazakii* ATCC 29544^T (encoding AmpC, which was designated CSA-1) = KF623543; *C. sakazakii* Su 92 (encoding AmpC, which was designated CSA-2) = KF640250; *C. malonaticus* LMG 23826^T (encoding AmpC, which was designated CMA-1) = KF640251; *C. malonaticus* Su126 (encoding AmpC, which was designated CMA-2) = KC665723.

FIG. 1. Analysis of the four *Cronobacter ampC*/AmpC sequences including those of the strains *Serratia marcescens* SST-1 (AB008455.1, BAA23131) and *Pantoea* sp. At-9b (YP_004115822.1). **(A)** Nucleic acid alignment of the 5′ untranslated (promoter) regions of the ATG start codon is written in bold. **(B)** Amino acid alignment of the translated CDS; the catalytic site including the active serin at amino acid position 80 as well as a second potential site at amino acid position 240 most likely representing two components of the catalytic triad are boxed. **(C)** Nucleic acid alignment of the 3′ untranslated region. TAA stop codon is written in bold.

A

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Pantoea sp. At9b (Yp_004115822.1) ATTGTAAGTC CCGCTGCGTC TCAGCGGCCA ATTACGCTTA CGCAGTTGTG AAGTTGTTCA
Serratia SST-1(AB008455.1)          AGAGCGTCGC GGCAGCCGTA AAGGAATGAC ATCATCAATC AGGGAAAGCG CCGTGCATAA
C. sakazakii ATCC 29544T            GCGATAACCC AAATCCGGCG AGAAAACGTC CTTTGTTAGG GAAAATAATG GAGTTTTGTC
C. sakazakii SU 92                  GCGATAACCC AAATTCGACG AGAAAACGTC CTTTGTTATG GAAAATTATG GCGATTTGTC
C. malonaticus LMG  23826T          GCGATAACCC AAATCCGACG AGAAAACGTC CTTTTTTATG GAAAATTATG GCGATTTGTC
C. malonaticus SU 126               GCGATAACCC AAATTCGACG AGAAAACGTC CTTTGTTATG GAAAATTATG GCGATTTGTC

Pantoea sp. At9b (Yp_004115822.1)      CTTAATTGAA AAAGGTTCTA TAGTGATGGC GGCACTCAAA ATAAAAGGAA TTTCAATG
Serratia SST-1(AB008455.1)             CGCCAATAAA AACTTTTGGC CGCGGCCGAT GCCCTGCAAC CGAAGAGCCT CTATCATG
C. sakazakii ATCC 29544T           ACAGTAAGCG GAGATAATGG CGGTTTGTGT GCGGTGAAAG AGGCGAGGAG AGCCGATG
C. sakazakii SU 92                  ACAGCAAACC GGGATAATGG CGGTTTGTGT GCGGTGAATG AGGCGAGGAG AGCCGATG
C. malonaticus LMG  23826T          ACAGCAAACC GGGATAATGG CGGTTTGTGT GCGGTGAATG AGGCGAGGAG AGCCGATG
C. malonaticus SU 126               ACAGCAAACC GGGATAATGG CGGTTTGTGT GCGGTGAATG AGGCGAGGAG AGCCGATG
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B

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                                        20                  40
Pantoea sp. At-9b (YP_004115822.1) MRLLTKLTLL LAWASGSALA QAPDIA---S ITRPLMQQYQ VPGMAVAVLY 47
Serratia SST-1 (BAA23131.1)        MTKMNRLAAA LIAALILPTA HAAQQQDIDA VIQPLMKKYG VPGMAIAVSV 50
C. sakazakii ATCC 29544T           MK--SKVSAL LMMIVLAGHA QAAPVAPPDA VVKPLMARYQ IPGMAVAVSV 48
C. sakazakii Su 92                 MK--SKVSAL LMMIVLAGHA QAAPVAPPDA VVKPLMARYQ IPGMAVAVSV 48
C.malonaticus LMG 23826T           MK--SKVSAL LMMIVLAGHA QAAPVAPPDA VVKPLMARYQ IPGMAVAVSV 48
C. malonaticus Su 126              MK--SKVSAL LMMIVLAGHA QAAPVAPPDA VVKPLMARYQ IPGMAVAVSV 48
                                    60                  80                  100
Pantoea sp. At-9b (YP_004115822.1) RGKTTFYNYG VASKTTQQPV TEQTLFEIGS LSKTFTATLA SYAVQQHKMR 97
Serratia SST-1 (BAA23131.1)        DGKQQIYPYG VASKQTGKPI TEQTLFEVGS LSKTFTATLA VYAQQQGKLS 100
C. sakazakii ATCC 29544T           NGETHFWHYG VASKATRKPV DENTLFEIGS LSKTFTATLA SKAQQDGKLD 98
C. sakazakii Su 92                 NGETHFWHYG VASKATRKPV DENTLFEIGS LSKTFTATLA SKAQQDGKLD 98
C.malonaticus LMG 23826T           NGETHFWHYG VASKATRKPV DEKTLFEIGS LSKTFTATLA SGAQHEGKLD 98
C. malonaticus Su 126              NGETHFWHYG VASKATRKPV DEKTLFEIGS LSKTFTATLA SAAQQEGKLD 98
                                                   120                 140
Pantoea sp. At-9b (YP_004115822.1) FSDSASQWLP ELKGSAFDHV SLLNLATHTS GMPLFVPDAV TNTAQLMAWY 147
Serratia SST-1 (BAA23131.1)        FNDPASRYLP ELRGSAFDGV SLLNLATHTS GLPLFVPDDV TDNAQLMAYY 150
C. sakazakii ATCC 29544T           FSAPASQYLP ALKGSAFDNV TLLNLATHTA GTPLFVPDAV KNTTQLMDWY 148
C. sakazakii Su 92                 FSAPASQYLP ALKGSAFDHV TLLNLATHTA GTPLFVPDAV KNTPQLMDWY 148
C.malonaticus LMG 23826T           FSAPASQYLP ALKGSAFDHV TLLNLATHTA GTPLFVPDAV KNTAQLMAWY 148
C. malonaticus Su 126              FSAPASQYLP ALKGSAFDHV TLLNLATHTS GTPLFVPDAV KNTAQLMAWY 148
                                    160                 180                 200
Pantoea sp. At-9b (YP_004115822.1) QQWQPPAVPG TQRVYSNLGI GMLGMIAAKS LHQPFRQAME QQLLPAMGMH 197
Serratia SST-1 (BAA23131.1)        RAWQPKHPAG SYRVYSNLGI GMLGMIAAKS LDQPFTQAME QGMLPALGMR 200
C. sakazakii ATCC 29544T           RAWQPEKPVG TERVYSNLGI GLLGMITAKA LDKPFSEAME QGLLRDFGMT 198
C. sakazakii Su 92                 RAWQPEKPVG TERVYSNLGI GLLGMITAKA LDKPFSEAME QGLLRDFGMT 198
C.malonaticus LMG 23826T           RAWQPEKPVG TERVYSNLGI GLLGMITAKA LDKPFSEAME QGLLKGFGMT 198
C. malonaticus Su 126              RAWQPEKPVG TERVYSNLGI GLLGMITAKA LDKPFSEAME QGLLRDFGMT 198
                                                   220                 240
Pantoea sp. At-9b (YP_004115822.1) HSWITVPSAR MQDYAQGYNK QDQPVRVTPG PLDAEAYGLK SSSADLIRWL 247
Serratia SST-1 (BAA23131.1)        HTYVQVPAAQ MANYAQGYNK DDKPVRVNPG PLDAESYGIK SNARDLIRYL 250
C. sakazakii ATCC 29544T           HTFINVPEAA MDNYAQGYNK DDKPVRVTPG PLDAESYGLK SGSADLLRYL 248
C. sakazakii Su 92                 HTFINVPDAA MGDYAQGYNK DDKPVRVTPG PLDAESYGLK SGSADLLRYL 248
C.malonaticus LMG 23826T           HTFINVPDAA MDDYAQGYNK DDKPVRVTPG PLDAESYGLK SGSADLLRYL 248
C. malonaticus Su 126              HTFINVPDAA MDDYAQGYNK DDKPVRVTPG PLDAESYGLK SGSADLLRYL 248
                                    260                 280                 300
Pantoea sp. At-9b (YP_004115822.1) AIQINAVKID PNWRQAIAAT HNGQYHTEAF TQAMMWEYYP LPVTESALVA 297
Serratia SST-1 (BAA23131.1)        DANLQQVKVA HAWREALTAT HVGYYKAGAF TQDLMWENYP YPVKLSRLIE 300
C. sakazakii ATCC 29544T           QIQLGEQEVA PGWRQAINAT HNGYYRSGEF TQGLMWEYYP WPSPLSRLVE 298
C. sakazakii Su 92                 QIQLGEQEVA PGWRQAINAT HNGYYRSGEF TQGLMWEYYP WPSPLSRLVE 298
C.malonaticus LMG 23826T           QIQLGEQEVA PGWRQAINAT HNGYYRSGEF TQGMMWEYYP WPSPLSRLVE 298
C. malonaticus Su 126              QIQLGEQEVA PRWRQAINAT HNGYYRSGEF TQGMMWEYYP WPSPLSRLVE 298
                                                   320                 340
Pantoea sp. At-9b (YP_004115822.1) GNSGQRIMQG MAAQAITPPQ PAPQQAWYNK TGSTNGFSTY AVFIPSQQVA 347
Serratia SST-1 (BAA23131.1)        GNNAGMIMNG TPATAITPPQ PELRAGWYNK TGSTGGFSTY AVFIPAKNIA 350
C. sakazakii ATCC 29544T           GNSSQRIMKG LAATAIVPPQ PAPQAAWYNK TGSTNGFSTY AVFIPEKRIA 348
C. sakazakii Su 92                 GNSSQRIMKG LATTAIVPPQ PAPQAAWYNK TGSTNGFSTY AVFIPEKRIA 348
C.malonaticus LMG 23826T           GNSSQRIMKG LAATAIVPPQ PAPEAAWYNK TGSTNGFSTY AVFIPEKRIA 348
C. malonaticus Su 126              GNSSQRIMKG LAATAIVPPQ PAPEAAWYNK TGSTNGFSSY AVFIPEKRIA 348
                                    360
Pantoea sp. At-9b (YP_004115822.1) VIMLANKWFP NDDRVVAVHK IIQAIQGKE*376
Serratia SST-1 (BAA23131.1)        VVMLANKWFP NDDRVEAAYR IVQALD-KR*378
C. sakazakii ATCC 29544T           LIMLANKWFP NDDRVKAAYT IIQELD--K*375
C. sakazakii Su 92                 LIMLANKWFP NDDRVKAAYA IIQELD--K*375
C.malonaticus LMG 23826T           LIMLANKWFP NDDRVKAAYA IIQQLD--K*375
C. malonaticus Su 126              LIMLANKWFP NDDRVKAAYA IIQQLD--K*375
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C

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Pantoea sp. At9b (Yp_004115822.1)  TAACCATGCA ATTACGTGCA TTACGCAGTG ACGATTTTCC CTTATGGCTG
Serratia SST-1(AB008455.1)         TGACAATCAG GGGCGCCGGC GGGGCGCCCC TA
C. sakazakii ATCC 29544T           TAACCCGTCA TAAAAAAGGC CGCCCGTGGG GCGGCCTTGT TGTGAATTAA
C. sakazakii SU 92                 TAACGCGCCA TAAAAAAGGC CGCCCGTGGG CGGCCTTGTT GTGAATCAAC
C. malonaticus LMG 23826T          TAACGCGCCA TAAAAAAGGC CGCCCGTGGG CGGCCTTGTT GTGAATCAAC
C. malonaticus SU 126              TAACGCGCCA TAAAAAAGGC CGCCCGTGGG CGGCCTTGTT GTGAATCAAC

Pantoea sp. At9b (Yp_004115822.1)  GCGTTGTGGC AGGGCTATCA GCGTTTTTAT GCCACCGCAA TCAACGAAGA
Serratia SST-1(AB008455.1)
C. sakazakii ATCC 29544T          GCGCTCAGAG CTGCGTTATC ACCAC
C. sakazakii SU 92                 GCGCTCAGAG CTGCGTGA
C. malonaticus LMG 23826T          GCGCTCAGAG CTGCGTGATC ACC
C. malonaticus SU 126              GCGCTCAGAG CTGCGTGATC ACC
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Results

Screening of 139 *Cronobacter* spp. isolates originating from a milk powder production facility as well as the two type strains for the species *C. sakazakii* and *C. malonaticus* for their antibiotic resistance suggested the presence of two different susceptibility patterns regarding the two beta-lactam antibiotics ampicillin and cephalothin in the two species: (i) amp^S/ceph^S and (ii) amp^S/ceph^R. The majority (87%) of the strains exhibited susceptibility to ampicillin but intermediate or full resistance to cephalothin. In the remaining strains, the amp^S/ceph^S phenotype was observed. In an effort to elucitate the molecular bases of this feature, four strains were selected for further experiments: *C. sakazakii* type strain 29544^T (amp^S/ceph^R), *C. sakazakii* isolate Su 92 (amp^S/ceph^S), *C. malonaticus* LMG 23826^T (amp^S/ceph^S), and *C. malonaticus* Su 126 (amp^S/ceph^R).

A database search was performed on the whole genome sequence available for *C. sakazakii* ES15 (GenBank: CP003312.1), and a coding sequence (CDS) annotated as putative beta-lactamase (*ampC*, GenBank: AFJ99606.1, genome position: 2.159.483–2.160.610) was identified. Based on these data, primers were designed spanning the CDS, including a putative promoter element. The putative *ampC* genes were successfully amplified in all four target strains, and amplicons were subjected to sequencing.

Deduced protein sequences revealed that the four *Cronobacter* AmpC showed the highest—although still relatively low—protein homology to AmpC in *Serratia marcescens* SST-1 (65–66% identity, BAA23131) and *Pantoea* sp. At-9b (68–69% identity, YP_004115822.1). In Fig. 1, the nucleic acid alignment of the 5′ untranslated (promoter) region (1A), the amino acid alignment of the translated CDS (1B), as well as the nucleic acid alignment of the 3′ untranslated region of the four *Cronobacter* AmpC sequences, including the *Serratia marcescens* SST-1 (AB008455.1, BAA23131) and *Pantoea* sp. At-9b (YP_004115822.1) (1C), is depicted.

The *ampC* CDS of the four *Cronobacter* strains originally exhibiting divergent cephalothin resistance patterns were cloned and expressed in *E. coli* DH5 alpha. Determination of the MICs by means of the E-tests revealed (although with minor variations) identical susceptibility patterns for all transformants, namely susceptibility to ampicillin and resistance to cephalothin, according to CLSI interpreting criteria (Table 1).

AmpC beta-lactamases are cephalosporinases from the functional group 1 and molecular class C in the Bush–Jacoby–Medeiros classification scheme of beta-lactamases.

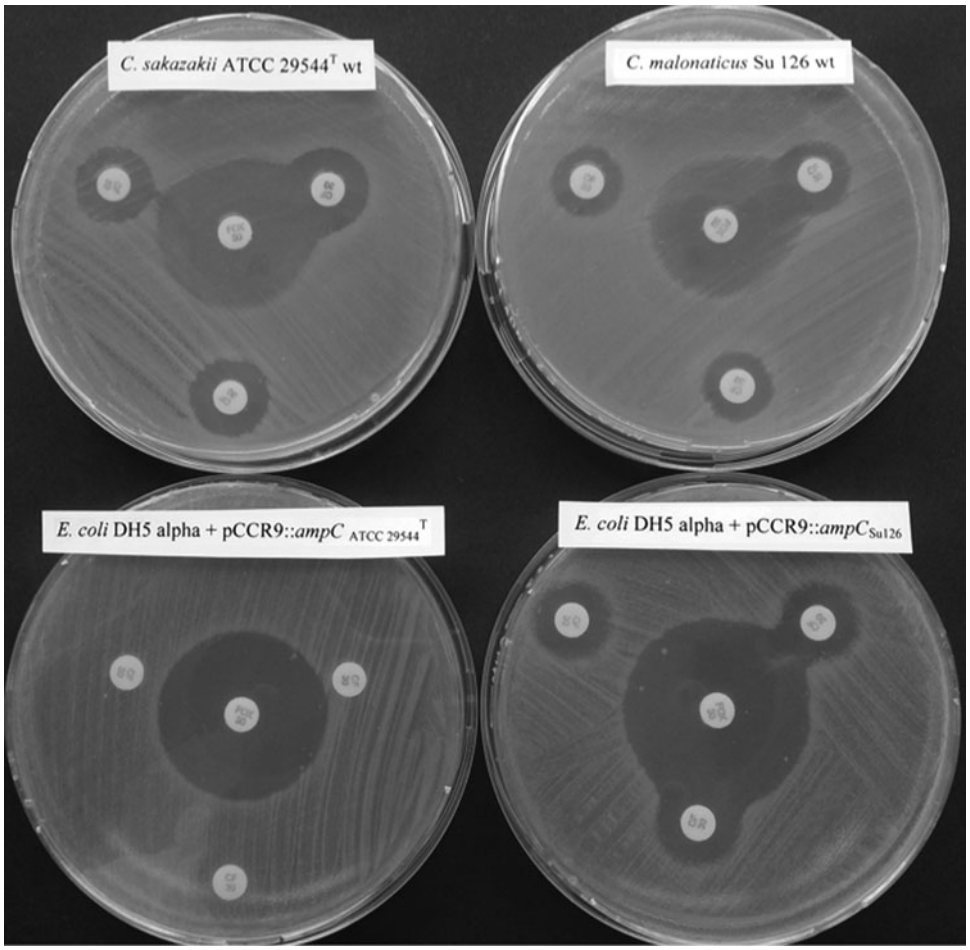

FIG. 2. Results of double disk inducer (cefoxitin) experiments on *C. sakazakii* ATCC 29544^T, *C. malonaticus* Su 126, and their respective *Escherichia coli* transformants. Inducer disk (Fox30) placed in the middle of each plate, and three synergy indicator disks (CF30) placed in the vicinity of Fox30 separated by distances of 10, 15, and 25 mm, respectively.

They are not inhibited by beta-lactamase inhibitors such as clavulanic acid. Additionally, certain beta-lactams such as cefoxitin, clavulanic acid, and carbapenems are strong AmpC beta-lactamase inducers. In order to test a potential induction effect possibly affecting the phenotype for cephalothin, double disk experiments were carried out using either amoxicillin/clavulanic acid or cefoxitin as inducers. For this purpose, the potential inducer disk was placed adjacent to three cephalothin disks, separated by distances of 10, 15, and 25 mm respectively, on Mueller Hinton agar. Figure 2 exemplifies the results of these experiments for the cefoxitin/cephalothin combination. No antagonistic effect was observed, as would have been expected in the case of induction. Results from analogous experiments using amoxicillin/clavulanic acid as an inducer were comparable (data not shown). In conclusion, the results showed that (i) *Cronobacter* wt and respective *E. coli ampC* transformants were fully susceptible to cefoxitin, and (ii) no induction effect was observed.

In many organisms, including *Enterobacter* spp., the expression/induction of the *ampC* appears to involve several gene products associated with this regulation. These gene products include AmpR, AmpD, and AmpG. Performing a GenBank homology search for these regulatory elements revealed that both *C. sakazakii* and *C. malonaticus* harbor homologues for AmpG and AmpD. However, no homologue could be identified for the key AmpC regulator AmpR in either of the two species (data not shown). This finding (*i.e.*, absence of *ampR*) is further evidence in support of our experimental observation of the *Cronobacter* beta-lactamase being noninducible.[22]

Discussion

The presence/expression of a beta-lactamase in *Cronobacter* has been discussed controversially in the literature. Several studies have suggested that *Cronobacter* is susceptible to beta-lactam antibiotics.[13,15,21] However, the presence of beta-lactamases in *Cronobacter* was confirmed in a study by Pitout et *al.*[17] who showed that *Cronobacter* strains sensitive to all cephalosporins, including cefoxitin, produced Bush group 1 beta-lactamases with isoelectric points of 7.4–8.0 at low levels. *Enterobacteriaceae* species, which naturally express their beta-lactamases at negligible or low levels, are not uncommon, and include several species (*e.g.*, *Shigella* spp.,[16] *Proteus* mirabilis,[11] and *Edwardsiella tarda*[20]).

We used a cloning approach in order to answer the question of the presence and the expression of a (AmpC) type beta-lactamase in *Cronobacter* isolates. This was a somewhat surprising result, as the strains were originally selected due to the observed differences in their resistance to cephalothin in their wild type background. The higher MIC values for ampicillin in the transformants may be explained as ''gene dosage effect,'' that is, copy (and thus the gene copy) number of the (low copy) pCCR9[18] vector versus single chromosomal copy. By this approach, we could show that *C. sakazakii* and *C. malonaticus* possess group C beta-lactamases[2] with a highly specialized phenotype. The sequences of the novel AmpC beta-lactamases were deposited in GenBank and designated as follows: CSA-1 (*C. sakazakii* ATCC 29544^T); CSA-2 (*C. sakazakii* Su 92); CMA-1 (*C. malonaticus* LMG 23826^T); and CMA-2 (*C. malonaticus* Su 126). The divergences in cephalothin MIC values, as observed before and after cloning of the *ampC* genes of *Cronobacter* strains exhibiting the amp^S/ceph^S phenotype, may be explained by the presence of yet unknown influencing factors present in the natural *Cronobacter* background, but absent in *E. coli*.

Disclosure Statement

No competing financial interests exist.

References

1. **Block, C., O. Peleg, N. Minster, B. Bar-Oz, A. Simhon, I. Arad, and M. Shapiro.** 2002. Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. Eur. J. Clin. Microbiol. Infec. Dis. **21:**613–616.
2. **Bush, K., and G.A. Jacoby.** 2010. Updated functional classification of β-lactamases. Antimicrob. Agents Chemother. **54:**969.
3. **Clinical Laboratory Standards Institute.** 2013. Performance standards for antimicrobial susceptibility testing: twenty-third informational supplement M100-S23. CLSI, Wayne, Pennsylvania.
4. **El-Sharoud, W.M., S. O'Brien, C. Negredo, C. Iversen, S. Fanning, and B. Healy.** 2009. Characterization of *Cronobacter* recovered from dried milk and related products. BMC Microbiol. **9:**24.
5. **Muytjens, H.L., H.C. Zanen, H.J. Sonderkamp, L.A. Kollée, I.K. Wachsmuth, and J.J. Farmer 3rd.** 1983. Analysis of eight cases of neonatal meningitis and sepsis due to *Enterobacter sakazakii*. J. Clin. Microbiol. **18:**115–120.
6. **Friedemann, M.** 2009. Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. Eur. J. Clin. Microbiol. Infect. Dis. **28:**1297–1304.
7. **Hunter, C.J., and J.F. Bean.** 2013. *Cronobacter*: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. J. Perinatology **33:**581–585.
8. **Iversen, C., N. Mullane, B. McCardell, B.D. Tall, A. Lehner, S. Fanning, R. Stephan, and H. Joosten.** 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii* and proposal of *Cronobacter sakazakii* gen. nov. comb. nov., *C. malonaticus* sp. nov., *C. turicensis* sp. nov., *C. muytjensii* sp. nov., *C. dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *C. dublinensis* sp. nov. subsp. *dublinensis* subsp. nov., *C. dublinensis* sp. nov. subsp. *lausannensis* subsp. nov., and *C. dublinensis* sp. nov. subsp. *lactaridi* subsp. nov. Int. J. Syst. Evol. Microbiol. **58:**1442.
9. **Joseph, S., E. Cetinkaya, H. Drahovska, A. Levican, J. Figueras, and S.J. Forsythe.** 2011. *Cronobacter condimenti* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp.genomospecies 1, recovered from a leg infection, water, and food ingredients. Int. J. Syst. Evol. Microbiol. doi:10.1099/ijs.0.032292-0.
10. **Lai, K.K.** 2001. *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and a review of the literature. Medicine (Baltimore) **80:**113–122.
11. **Livermore, D.M.** 1995. Beta-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
12. **Reij, M.W., I. Jongenburger, E. Gkogka, L.G. Gorris, and M.H. Zwietering.** 2009. Perspective on the risk to

infants in the Netherlands associated with *Cronobacter* spp. occurring in powdered infant formula. Int. J. Food Microbiol. **136:**232–237.

13. **Muytjens, H.L., and R. van de Ros-van de Repe.** 1986. Comparative *in vitro* susceptibilities of eight *Enterobacter* species, with special reference to *Enterobacter sakazakii*. Antimicrob. Agents Chemother. **29:**367–370.
14. **Müller, A., R. Stephan, C. Fricker-Feer, and A. Lehner.** 2013. Genetic diversity of *Cronobacter sakazakii* isolates collected from a Swiss infant formula production facility. J. Food Prot. **76:**883–887.
15. **Nazarowec-White, M., and J.M. Farber.** 1999. Phenotypic and genotypic typing of food and clinical isolates of *Enterobacter sakazakii*. J. Med. Microbiol. **48:**559–567.
16. **Normark, S., T. Grundstrom, and S. Bergstrom.** 1980. Susceptibility to penicillins and cephalosporins in beta-lactamase producing strains of *E. coli* and relative amount of beta-lactamase produced from these strains. Scand. J. Infect. Dis. Suppl. **25:**23–29.
17. **Pitout, J.D., E.S. Moland, C.C. Sanders, K.S. Thomson, and S.R. Fitzsimmons.** 1997. Beta-lactamases and detection of beta-lactam resistance in *Enterobacter* spp. Antimicrob. Agents Chemother. **41:**35–39.
18. **Randegger, C.C., A. Keller, M. Irla, A. Wada, and H. Hächler.** 2000. Contribution of natural amino acid substitutions in SHV extended-spectrum beta-lactamases to resistance against various beta-lactams. Antimicrob. Agents Chemother. **44:**2759–2763.
19. **Rej, M.W., I. Jongenburger, E. Gkongka, L.G. Gorris, and M.H. Zwietering.** 2009. Perspective on the risk to infants in the Netherlands associated with *Cronobacter* spp. occurring in powdered infant formula. Int. J. Food Microbiol. **136:**232–237.
20. **Stock. I., and B. Wiedemann.** 2001. Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri* and E. *hoshinae*. Antimicrob. Agents Chemother. **45:**2245–2255.
21. **Stock, I., and B. Wiedemann.** 2002. Natural antibiotic susceptibility of *Enterobacter amnigenus*, *Enterobacter cancerogenus*, *Enterobacter gergoviae* and *Enterobacter sakazakii* strains. Clin. Microbiol. Infect. **8:**564–578.
22. **Sykes, R.B., and M. Matthew.** 1976. The beta-lactamases of gram-negative bacteria and their role in resistance to beta-lactam antibiotics. J. Antimicrob. Chemother. **2:** 115–157.

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